Docket No.:

IN THE UNITED STATES PATENTS AND TRADE MARK OFFICE

Applicant:	Susan J Clark *et al.*
Serial number:	09/673,448
Filing date:	November 27, 2000
Title:	Assay for Methylation of GST-Pi gene
Examiner:	Jeanine Goldberg
Group Art Unit:	1634

DECLARATION UNDER 37 C.F.R. 1.132

I, Peter Laurence Molloy of 41 Bellevue St, Chatswood, NSW 2067, Australia, declare that:

1. My qualifications and relevant research experience are set out in my Curriculum Vitae annexed hereto and marked Annexure A.

2. I am a co-applicant and co-inventor of US Patent Application Serial Number 09/673,448 ("the present application") filed and am accordingly familiar with the invention described and claimed in the present application.

3. I have read and understood the Office Action mailed July 6, 2004 ("the Office Action") in connection with the present application. I am advised that the matters raised in paragraphs 1-10 of the Office Action will be attended to by way of submission and/or appropriate amendment of the claims in question.

5. In this my declaration, I will directly address the matters raised in paragraphs 11,12 and 13 of the Office Action, where the Examiner rejects the claims remaining in this application on the ground of obviousness.

6. For convenience, I set out below the first paragraph of paragraph 11 of the Official Action:

Claims 1-14, 17-25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Lee *et al.* (Cancer Epidemiology, Biomarkers, Prevention. Vol 6, pages 443/450, June 1997) in view of Herman *et al.* (US Pat. 5,786,146, July 1998).

6. In rejecting these Claims as obvious in the light Lee *et al.* 1997 (Cancer Epidemiology, Biomarkers, Prevention. Vol 6, pages 443/450, June 1997) in view of Herman *et al.* (US Pat. 5,786,146), I believe that the Examiner has made a number of assumptions about the general state of prior knowledge and specifically what has been demonstrated in the cited Prior Art which I believe are inconsistent with my understanding of what was known to those working in the field just before the priority date of the present application.

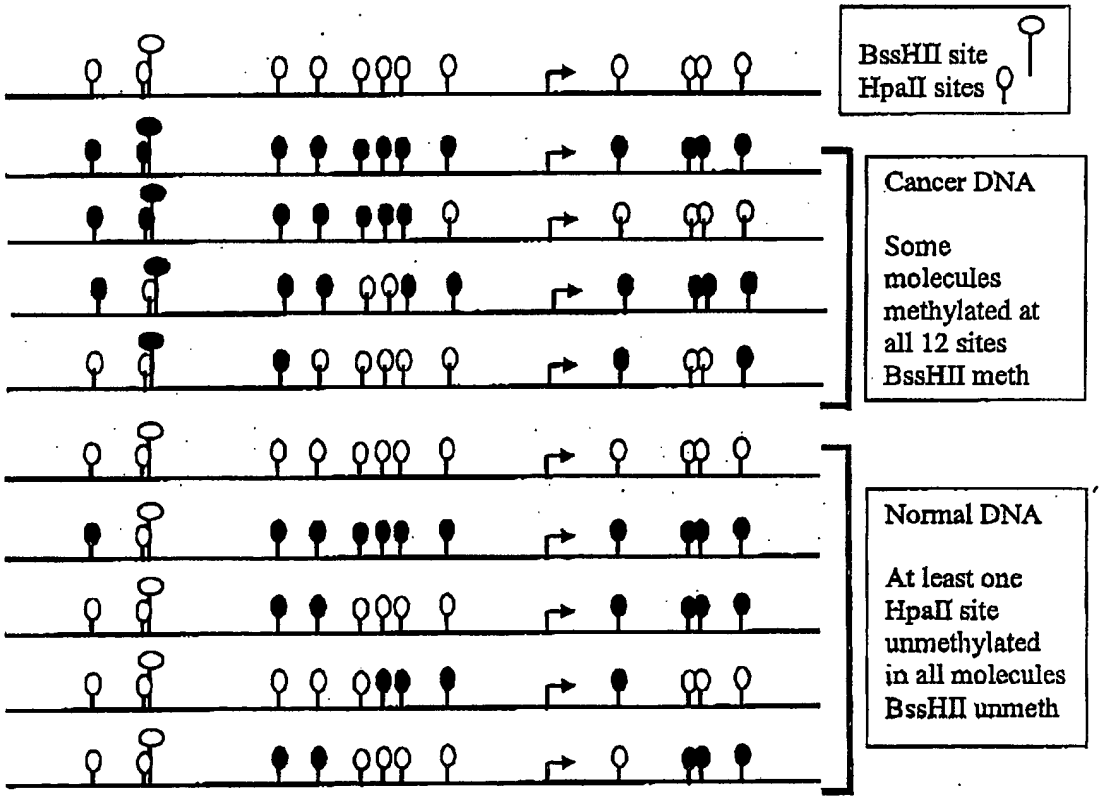
7. I regard that the key discovery of the invention described in the present application is the different profiles in cancer and normal tissue of methylation across individual CpG sites in the promoter and within the transcribed region of the *GSTP1* gene that allow the development of assays for cancer detection targeted to these methylation differences. The discovery includes the extent of the region useful for such assays as well as comparative information on each CpG site that can be used to optimise sensitivity and specificity of assays.

8. Lee *et al.*. 1994 (Lee *et al.*, PNAS 91: 11733-11737 (1994)) and *Herman et al.* demonstrated that a BssHII site in the *GSTP1* gene promoter (site at base -298, includes CpG sites -34 and -35) is not detectably methylated in normal tissues but is significantly methylated in 20 of 20 cancer specimens studied. Methylation at two further restriction enzyme sites (NotI, CpG sites -17 & -18, and SacII, CpG sites -13 & -12) in the promoter is demonstrated in a prostate cancer cell line.

9. In the further publication Lee *et al.* 1997, demonstrated that for 52 of 57 prostate cancer DNA samples a 605bp PCR fragment extending from 408 bases upstream to 197 bases downstream of the transcription start site could be amplified following digestion of the DNA with HpaII. 3/37 matched normal specimens showed evidence of methylation (probably contaminating cancer cells). 20 samples of seminal vesicle DNA showed no amplification.

The amplification of the 605 bp fragment requires that some DNA molecules are methylated at all 12 HpaII sites. Lack of amplification requires that at least one of the 12 sites is unmethylated and so the DNA is cut. This knowledge does not add further to our understanding of which CpG sites show differential methylation between cancer and normal tissue – only those sites contained within the BssHII, NotI and SacI sites contained within the promoter and no sites within the transcribed region of the gene had been shown to be differentially methylated and so suitable for application of the method of Herman *et al.*

10. I believe that prior to the disclosure of the present application, those working in the field understood that there were a range of possible differences in methylation between normal tissue and prostate cancer tissue as shown diagrammatically and discussed below:



11. In cancer Lee *et al.* 1997, Lee and co-workers had established that in a fraction of the DNA, all 12 HpaII sites within the amplified region were methylated. Some, or most, molecules may have been methylated at only a subset of these 12 sites. In DNA from normal tissue, the BssHII site was not detectably methylated and no molecules could be detected in which all 12 HpaII sites were methylated. The nature of methylation at individual sites was not characterised. Some of the range of possible profiles are shown in the diagram above.

12. It appears to me that the Examiner's conclusion on obviousness is based on the assumption that if one or certain sites in a promoter region or CpG island are methylated, then all will be (and conversely if some sites are unmethylated, then all will be). However, a range of data available at that time from methylation sequence analysis of genes associated with cancer development, imprinted genes and tissue-specific genes had demonstrated that this was not generally understood to be the case (though it appeared to be in the specific case of genes on the inactive X chromosome).

13. I agree with statement made in the paragraph bridging pages 2 and 3 of the present application that:

> "Such studies have indicated that, while a population of molecules may conform to an overall pattern of methylation, not all molecules will be identical and methylation may be found on only a fraction of the molecules at some sites (13, 16)."

14. Moreover the references Stirzaker *et al.* (reference 8) and Tremblay *et al.* (reference 9) are cited in the present application as examples where cytosine methylation had been examined at all cytosine bases using genomic sequencing methods, both display substantial heterogeneity of methylation at different CpG sites. For example, see Figures 5, 6 and 7 of Stirzaker and Figures 2 to 6 of Tremblay *et al.*

15. In addition to papers cited in the patent application, numerous other papers had reported on the heterogeneity of DNA methylation profiles and regional-specific methylation changes in cancer and other cell types. Examples include:

1. Desiderato L, Davey MW and Piper AA (1997) Demethylation of the human MDR1 5' region accompanies activation of P-glycoprotein expression in a HL60 multidrug resistant subline. Somatic Cell Mol Genet. 23:391-400

> "...we demonstrated that HL60 DNA is methylated at multiple sites within two distinct areas, one upstream and one downstream of the transcription start point. Only a single site in each area was methylated in all strands examined, with the remaining adjacent sites showing partial methylation. ..." (Abstract, page 391)

Restricted, localised methylation within a CpG island is demonstrated (see Figure 4)

2. Watts GS, Pieper RO, Costello JF, Peng Y-M, Dalton WS and Futscher BW (1997) Methylation of discrete regions of the O6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. Molec Cell Biol 17:5612-5619.

> "Bisulfite sequencing of the MGMT CpG island promoter revealed large increases in the levels of CpG methylation within discrete regions of the 8226/V MGMT CpG island compared to those in 8226S."

Variations from 0 to 100% methylation are demonstrated at different CpG sites across the CpG island (Figure 3).

(similar data for MGMT gene in Qian XC and Brent TP (1997) Cancer Res 57:3672-3677)

3. Stoger R, Kajimura TM, Brown WT and Laird CD (1997) Epigenetic variation illustrated by DNA methylation patterns in the fragile-X gene FMR1. Human Molec Genet 11:1791-1801.

Studies of variation in methylation patterns in and between individuals over time (eg Figure 4).

4. Feil R, Walter J, Allen ND and Reik W (1994) Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. Development 120:2933-2943.

Diverse patterns of methylation seen on individual molecules in Igf2 upstream and promoter 2 regions (Figure 2).

5. Salvatore P, Benvenuto G, Caporaso M, Bruni CB and Chiariotti L (1998) High resolution methylation analysis of the galectin-1 gene promoter region in expressing and non-expressing tissues. FEBS Lett 421:152-158.

Show heterogeneous methylation across galectin-1 promoter, expression correlating with density of methylation rather than specific sites (Figures 2, 3 & 4)

6. Leegwater PA, Lambooy LH, De Abreu RA, Botternik JP, van den Heuvel LP (1997) DNA methylation patterns in the calcitonin gene region at first diagnosis and at relapse of acute lymphoblastic leukemia (ALL). Leukemia 11:971-978.

"There are marked regional differences in the frequency of methylation of individual CpG sites and the frequency of alterations between the two stages." (see Figures 3 and 4)

7. Hakkarainen M, Wahlfors J, Myohanen S, Hiltunen MO, Eskelinen M, Johansson R and Janne J. (1996) Hypermethylation of calcitonin regulatory gene sequences in human breast cancer as revealed by genomic sequencing. Int J Cancer 69:471-474.

Methylation differences between breast carcinomas and benign tumours are shown across the calcitonin gene promoter. The wide variation at individual CpG sites in both cancer and benign tissue is shown in Figure 4.

Earlier data from restriction enzyme analysis had also demonstrated differential methylation of neighbouring sites, eg.

8. Silva AJ, Ward K and White R (1993) Mosaic methylation in clonal tissue. Dev Biol 156:391-398

They particularly studied the YNZ22 locus in Leiomyomas and the IGH locus in colon and several tumour tissues and found that methylation at neighbouring CpG sites was independently regulated.

"Despite their clonal origin and their histological homogeneity, our analysis of several of these tumours has indicated that they are not homogeneously methylated at seven loci studied (JC767, YNH24, MCOC12, YNZ22, IGH, Hras1, RMU3)."

16. If, for arguments sake, the ordinary artisan were to apply the methylation specific PCR (MSP) method of Herman *et al.* to the detection of methylated *GSTP1* sequences in Lee *et al.* 1997, I believe that such application would be compromised by the lack of knowledge of which regions and specific CpG sites provided clear discrimination between cancer and normal tissue DNA. Also, as I have already indicated above, Lee *et al.* 1997 describe a method in which amplification requires that all 12 specified HpaII sites are methylated and where lack of amplification requires that at least one of the 12 specified sites is unmethylated (and so the DNA is cut prior to PCR). At page 13 of the Official Action, the Examiner states:

> "The response points out, correctly, that the primers need to be in close proximity to the CpG sites. The ordinary artisan would have clearly recognised this aspect of the MSP method from the teachings of *Herman et al.* Moreover the teachings of Lee include methylation CpG sites over a large region. The ordinary artisan would have recognized that several different amplifications may need to be performed to analyze the complete region." (My emphases)

In my view, if the ordinary artisan were to apply the MSP method of *Herman et al.* to the teachings of Lee *et al.* 1997, the ordinary artisan would have concluded that several amplifications would have been required to examine all 12 CpG sites. It could not be automatically assumed that the MSP method could be applied to any subset of the HpaII sites in order to provide an assay that discriminated cancer from normal

DNA. That is, the teachings of Herman *et al.* in regard to the use of the MSP method would not have altered the ordinary artisan's understanding of the teaching of Lee *et al.* that in order to establish the presence of prostate cancer, all 12 CpG sites specified in Lee *et al.* 1997 have to be shown to be methylated.

17. The requirement for all 12 CpG sites to be methylated for an accurate diagnosis of prostate cancer is reinforced by the indication in the paragraph bridging pages 448-449 of Lee *et al.* that:

> "... Perhaps a PCR assay for *GSTPI* CG island methylation changes similar to the one presented in this report may find use as a molecular staging of diagnostic test for prostate cancer. For such a test, careful attention will be required to reduce false positive and false negative results. ... In future, assay false positives and false negative test results may be monitored for quality control by the use of internal standards (e.g., standard DNA samples containing modified *GSTPI* promoter target sequences with fewer number of HpaII sites to monitor false positives) added to PCR mixtures. ..."

18. Furthermore, I note the Examiner's comment (page 13, last line to page 14, line 6) that *Herman et al.* does not solve the problem of multiple CpG sites by using primers of several hundred nucleotides in length and that a single primer is not used to distinguish all recognition sites. Rather, application of *Herman et al.* relies on the use of various primer pairs to distinguish all recognition sites in a promoter region.

19. In contrast, the present invention provides a diagnostic of prognostic assay for prostate cancer or liver cancer that does not require several different PCR amplifications. In order to predictably apply the MSP method without several amplification steps, the ordinary artisan would have had to been aware of the existence of CpG sites that show consistent differences between cancer and normal tissue and can be used to design effective MSP primers that cover a sufficient number of CpG sites (eg 2 to 4 CpG sites) known to be differentially methylated in cancer and normal DNA.

20. The most preferable sites for primer design in order to develop assays of maximal sensitivity and specificity are those that show the greatest level of methylation within

cancers and are most consistently methylated in different cancers, while being unmethylated in DNA derived from normal tissue.

21. If cancer-derived DNA showed uniform methylation across all CpG sites and normal DNA a lack of methylation across all CpG sites, then any sites could be chosen. However from the examples of scientific literature discussed in paragraphs 14 and 15 above, and the data incorporated in the present specification, it is clear that this is not the case. From the data of Lee *et al.* (1994 and 1997) neither the profile of methylation and preferred sites for discriminating cancer from normal DNA (except for the BssHII site), nor the boundaries of the region where differential methylation distinguished cancer and normal DNA were known, nor could they have been predicted.

22. The invention described in the present application is based on our discovery of the extent and specific pattern of methylation of CpG sites both upstream of the transcription start site and within the *GSTP1* gene in cancer and normal DNA that allow the development of assays to provide optimal sensitivity for detection of cancer DNA while minimising detection of DNA derived from normal cells. I therefore believe that the assay of the present invention in both new and would not have been obvious to the ordinary artisan working in the field before the priority date of the present application.

23. An additional discovery of the inventors of the present invention is that an assay for cancer may be based on determination of methylation of CpG sites within the transcribed region of the *GSTP1* gene.

24. Based on the prior art, it was predictable that sequences within the transcribed region would <u>not</u> discriminate cancer and normal DNA; it was indicated in Herman *et al.* that differences did not extend within the gene and that intragenic sequences were methylated in both expressing and non-expressing cells:

> "Furthermore, Southern blot analysis of prostate cancer cell line DNAs digested with the isoschizomers MspI and HpaII, which have several recognition sites distributed throughout the GSTP1 gene, revealed that the correlation of cytosine

hypomethylation with increased GSTP1 appeared to be restricted to cytosine residues present in 5' regulatory sequences (Fig.3, C and D)."

25. I therefore believe that our discovery that CpG sites from +1 to +53 could be used to discriminate cancer and normal DNA is new and would not have been obvious to the ordinary artisan.

26. In paragraph 12 of the Official Action, the Examiner concedes that neither Lee nor Herman *et al.* teach the specific primers for the amplification of the CpG island of the *GSTP1* gene. However, the examiner cites Jhaveri *et al.* as teaching the regions of GST-Pi which are methylated. In fact Jhaveri teaches that in two breast cancer cell lines three sites in the promoter region show methylation – the BssHII, NotI and SacII sites reported by Lee *et al.* (1994) for LNCaP prostate cancer cells (though the NotI site is substantially unmethylated in the ZR-75B line). They describe the extent of the CpG island and its potential for differential methylation, but provide no data as to the extent of its methylation in breast cancer cell lines or tissue or as to what sites/regions are methylated in normal tissue. It thus provides no further guide as to sites that can be used to discriminate between normal and cancer cells. As is clear from the examples cited above, methylation of a CpG island does not occur in an all or none manner.

27. In relation to the potential regional nature of methylation, the Examiner suggests that it would be a simple and routine matter to test a variety of primers for MSP until a satisfactory pair was found. In my opinion, this is certainly not a normal or routine task; many forward and reverse primers would need to be designed based on available CpG sites that were in sufficient proximity to enable primers with two or more CpG sites and then multiple MSP assays optimised for detection using known methylated DNA (and unmethylated DNA). To determine which assays were clinically useful would then require evaluation on sets of prostate cancer and normal prostate tissue as well as other tissues, blood etc. that might be encountered in clinical practice. An example demonstrating that not all MSP primer sets designed to the *GSTP1* sequences provide sufficient discrimination between cancer and normal DNA is that of Figure 4A, Panel A of the present application, where non-cancer DNA was detected in a number of tracks.

28. In paragraph 13 of the Official Action, the Examiner rejects claims 30-34 as being obvious over Lee *et al.* 1997, Herman *et al.* and Tchou *et al.* (Hepatology, Vol. 28, No. 4, pages 47, October 1998). With regard to the Tchou reference, I believe that the Provisional application, from which the present application claims priority, discloses the use of GSTP1 methylation assays for detection of liver cancer: see Fig 4C, Panel C (note incorrectly described as Panel B in text) and the text on page 22 lines 24-26 where GST methylation which provides results for a liver carcinoma cell line, HepG2. There is also reference at pages 10 and 21 to normal liver DNA that contains a limited amount of methylation near the transcription start site. I therefore believe that the claims of the present application relating to use of the assay of the invention for the detection of liver cancer are entitled to a priority date of the filing date of the provisional application, which predates the publication date of the Tchou reference. It therefore appears that the Tchou reference is not a prior art document for consideration of obviousness. As noted by the Examiner, both Lee *et al.* 1997 and Herman *et al.* are silent with regard to liver cancer.

29. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of this application or any patent issuing therefrom.

Dated this 21st day of December 2004.

P L O'Molloy

Peter Laurence Molloy

Annexure A

Qualifications and Experience of Dr Peter L Molloy

Qualifications
B. Sc (Hons) 1972, Monash University
PhD 1975, Monash University, Dept. of Biochemistry

Appointments

1975-1978	Postdoctoral Fellow (Damon Runyon-Walter Winchell Fellowship) Yale University, Dept of Human Genetics
1978-1981	Queen Elizabeth II Fellow, Dept of Biochemistry, University of Adelaide
1981-present	Research Scientist, CSIRO Molecular Science

Experience
I have over 30 years experience in molecular genetics, covering a diverse range of fields including yeast and human mitochondrial genetics, plant virus gene structure, chicken gene structure and mapping and extensive experience in mammalian gene regulation (promoters, transcription factors, chromosomal proteins, DNA methylation, gene therapy). A significant fraction of my research has involved studies of DNA methylation and its role in gene regulation (Publications 16, 21, 22, 28, 30, 41, 49, 50, 52, 58, 60 & 62 below) and I have extensive experience in the use of bisulphite treatment for analysis of DNA methylation, being a co-author on the original paper, Frommer *et al.*, describing its application for determination of sites of DNA methylation. .

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CpG Site Number	Restriction Site	Base Number of C of CpG site Relative to transcription start site	Base Number of C of CpG site as in Genbank ref M244485
-56		-705	520
-55	HpaII	-694	531
-54		-674	551
-53		-635	590
-52		-613	612
-51		-584	641
-50		-577	648
-49		-560	665
-48		-557	668
-47		-551	674
-46		-549	676
-45		-521	704
-44		-511	714
-43		-389	836
-42		-357	868
-41	HpaII	-342	883
-40		-336	889
-39		-331	894
-38		-310	915
-37		-308	917
-36	HpaII	-300	925
-35	BssHII	-297	928
-34	BssHII	-295	930
-33		-287	938
-32		-282	943
-31		-273	952
-30		-268	957
-29		-231	994
-28	HpaII	-197	1028
-27		-187	1038
-26		-185	1040
-25		-182	1043
-24	HpaII	-176	1049
-23		-162	1063
-22		-152	1073
-21		-148	1077
-20		-145	1080
-19	HpaII	-141	1084
-18	NotI	-131	1094
-17	NotI	-127	1098
-16	HpaII	-124	1101

-15	HpaII	-112	1113
-14		-109	1116
-13	SacII	-101	1124
-12	SacII	-99	1126
-11		-81	1144
-10	HpaII	-77	1148
-9		-74	1151
-8		-71	1154
-7		-53	1172
-6		-48	1177
-5		-43	1182
-4		-22	1203
-3		-15	1210
-2		-13	1212
-1		-4	1221
1		8	1232
2		11	1235
3		14	1238
4		23	1247
5		38	1262
6		40	1264
7		42	1266
8		47	1271
9	Note: extra G relative to M244485 creates CpG	49	1273
10	HpaII	55	1278
11		93	1316
12	HpaII	107	1330
13	HpaII	115	1338
14		128	1351
15		137	1360
16	HpaII	144	1367
17		149	1372
18		171	1394
19		183	1406
20		189	1412
21	HpaII	205	1428
22		210	1433
23		215	1438
24	HpaII	233	1456
25		239	1462
26		259	1482
27		266	1489
28		268	1491
29	HpaII	286	1509
30		312	1535
31		323	1546
32		333	1556

33		352	1575
34		417	1640
35	HpaII	425	1648
36		435	1658
37		453	1676
38		456	1679
39	HpaII	472	1695
40		492	1715
41		501	1724
42		517	1740
43		541	1764
44	HpaII	589	1812
45		599	1822
46		604	1827
47		610	1833
48	HpaII	615	1838
49		629	1852
50		645	1868
51		652	1875
52		657	1880
53		659	1882
54		667	1890
55		717	1940
56		725	1948
57		756	1979
58	HpaII	772	1995
59		819	2042
60		826	2049
61		876	2099
62		900	2123
63		937	2160
64		949	2172
65	HpaII	1010	2233
66		1039	2262
67		1082	2305
68		1198	2421
69		1284	2507
70		1294	2517
71		1296	2519
72		1376	2599
73		1379	2602
74		1392	2615
75		1638	2861
76		1651	2874